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TITLE: A Novel Differentiation Therapy Approach To Reduce The Metastatic Potential Of Basal, Highly Metastatic, Triple-Negative Breast Cancers

PRINCIPAL INVESTIGATOR: Isabel M. Chu, Ph.D.

CONTRACTING ORGANIZATION: The Geneva Foundation
Tacoma, WA 98402

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14. ABSTRACT

Low-grade breast cancer is associated with increased differentiation and reduced metastases, suggesting that reprogramming tumor cells to a more differentiated state could improve outcome. Utilizing a *novel differentiation therapy approach*, We have reprogrammed aggressive, basal, triple-negative Breast Cancer (BrCa) (generally with poor prognosis) towards a less aggressive phenotype by manipulating expression of the key mammary luminal differentiation transcription factor, GATA3. GATA3 is essential for programming undifferentiated mammary cells into a luminal subtype while myoepithelial/basal cells fail to express GATA3. Significantly, GATA3 expression is highly correlated with the luminal, more differentiated BrCa phenotype. We hypothesized that ectopic expression of GATA3 in metastatic, basal BrCa cell lines will reprogram them to a more differentiated, less metastatic phenotype. Over-expressing GATA3 in human basal-type MDA-MB-231 (231-GATA3) breast cancer cells induced significant morphological changes in 2- and 3-D cultures compared to control cells (231-Empty). 231-Empty cells maintained a spindle, elongated morphology, while 231-GATA3 cells became rounded and larger. In 3D Cultrex, 231-GATA3 cells appeared smaller, more organized, and rounded compared to 231-Empty cells. Microarray profiling of 231-GATA3 vs. 231-Empty cells revealed gene expression changes associated with increased adhesion, reduced extracellular matrix remodeling factors and reduced metastasis. Western blot confirmed reexpression of E-cadherin and an increase in cytokeratin-18 in 231-GATA3 cells, indicative of a more luminal phenotype. 231-GATA3 cells showed reduced LOX expression by real time PCR and knock down of GATA3 in the luminal BT474 cell line increased LOX expression. This demonstrates for the first time that GATA3 is an important differentiation factor that reduces the metastatic potential of MDA-MB-231. Ultimately, these findings suggest that transcription factor-induced differentiation pathways may be potentially novel therapeutic molecular targets to inhibit metastatic disease progression in combination with standard therapeutic treatments.

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INTRODUCTION

Although primary tumors in cancer patients are often successfully treated, the emergence of metastases generally heralds a poor prognosis (Gupta *et al.*, 2006). Metastatic disease is responsible for over 90% of cancer patient deaths (Gupta *et al.*, 2006). High-throughput gene expression profiling and molecular subtype clustering have been highly effective for predicting the propensity of a breast tumor to metastasize resulting in poor patient outcome. Based on hierarchical clustering analyses, breast tumors can be classified into distinct subtypes (Basal-like A and B, ErbB2+, Normal Breast-like, Luminal A, B and C) (Sorlie *et al.*, 2003; Hennessy *et al.*, 2009). Patients with basal-type tumors lacking estrogen receptor (ER), progesterone receptor (PR), and ErbB2 - referred to as basal triple-negative breast cancer (BTNBC) - have a worse prognosis compared to patients with more differentiated, less metastatic tumors expressing markers of the luminal lineage, including the transcription factors GATA3 and ER (Neve *et al.*, 2006; Perou *et al.*, 2000; Sorlie *et al.*, 2003). Expression of GATA3 is intimately associated with the luminal subtype of breast cancer and its expression is highly correlated with ER expression and many genes associated with the luminal subtype (Perou *et al.*, 2000; Usary *et al.*, 2004; Sorlie *et al.*, 2003). GATA3 is generally absent or minimally expressed in basal subtypes of breast cancer including MB231 cells. Furthermore, during mammary gland development, GATA3 expression was found essential for luminal differentiation (Kouros-Mehr *et al.*, 2006; Asselin-Labat *et al.*, 2007). Thus, we investigated the potential anti-tumorigenic role of GATA3 to reprogram BTNBC towards a more luminal and less aggressive phenotype.

BODY

For the first year, I proposed to address task 1a, part of task 1b and 1c. I have described below a detail description of the experiments and results obtained as I was addressing these tasks.

GATA3 reprograms MB231 cells to a more luminal subtype with a concomitant reduction in expression of metastasis-associated genes (task 1a)

We transduced MB231, GATA3 negative cells with lentivirus expressing GATA3-IRES-eGFP (231-GATA3), sorted for GFP-expressing cells and demonstrated that the GATA3 protein was ectopically expressed in 231-GATA3 cells. We performed gene expression profiling analyses to determine the effect of GATA3 expression on the transcriptome of MB231 cells. The expression of 1273 probe sets were found to be altered between 231-GATA3 and control 231-Empty cells (776 up- and 497 down-regulated in 231-GATA3 cells with fold change ≥ 1.5 and $p < 0.001$, false discovery rate (FDR) reaches 3%. A previous study of 51 human breast cancer cell lines identified a 305 gene signature that distinguishes the cells into Luminal, Basal A or Basal B subtypes (Neve *et al.*, 2006). Analysis using the Ingenuity software, revealed several network of genes altered upon GATA3 overexpression (Figure 1). We applied a gene centering method with z-score conversion of our microarray data combined with similarly converted data from Neve *et al.* dataset to perform hierarchical clustering of all of the cell lines using 249 unique signature genes available from both platforms. As demonstrated in Figure 2A, 231-Empty cells, as expected, clustered within the highly invasive basal B subtype, whereas the 231-GATA3 cells clustered within the luminal subtype (Figure 2A). GATA3 reduced the expression of 82 genes associated with the basal phenotype and increased the expression of 48 genes associated with the luminal phenotype. Among the genes up-regulated by GATA3 expression (also confirmed by Q-RT-PCR) were members of the claudin family, claudin 3 and claudin 4, whose low expression is characteristic of the claudin-low subtype of breast cancer (Hennessy *et al.*, 2009) (Figure 2B). Interestingly, the expression pattern of 231-Empty cells showed some differences compared to the MB231 cells used in the Neve *et al.* dataset. This may be due to clonal differences between the MB231 cells used by Neve *et al.* and our lab, or as a result of the transduction of the MDA-MB-231 cells with the GFP expressing lentivirus.

In order to validate that GATA3 did alter the expression of the Neve *et al.* Basal-Luminal signature genes along the luminal pathway, we performed Q-RT-PCR for several genes that distinguish the Luminal, Basal A and Basal B phenotypes (ANK3, CLDN3, CLDN4, KRT19, EPCAM, TSPAN13, ERBB3, FSCN1 and HMGA2). Our results confirmed that GATA3 altered the expression of these signature genes towards the luminal phenotype (Figure 2B). Consistent with the transition of 231-GATA3 cells to express genes associated with a more luminal phenotype. We also observed increased protein expression by Western blot of cytokeratin-18 and re-expression of E-cadherin in 231-GATA3 (Figure 2C). Altogether, these findings suggest that the transcription factor, GATA3 alone is sufficient to promote critical global changes resulting in the reprogramming and differentiation of the Basal B MB231 cells towards a more luminal, less aggressive phenotype.

Ectopic expression of GATA3 in MB231 alters cell morphology and cytoskeletal organization in 2D and 3D cultures (Task 1b)

As we outlined in task1b and given the critical role of GATA3 in luminal differentiation during mammary gland development (Kouros-Mehr *et al.*, 2006; Asselin-Labat *et al.*, 2007) and the strong association of GATA3 with the luminal breast cancer subtype (Perou *et al.*, 2000), we investigated whether the ectopic expression of GATA3 could alter the biological phenotype of the highly invasive and metastatic MB231 BTNBC cell line (Neve *et al.*, 2006) and reduce its metastatic potential. GATA3 induced significant morphological changes in MB231 cells both in 2D and 3D cultures compared to the control 231-Empty cells. 231-Empty cells maintained a spindle, elongated morphology, whereas 231-GATA3 cells were larger and cuboidal in shape when grown in 2D culture (Figure 3A). These findings are consistent with those of Yan and colleagues (Yan *et al.*, 2010) who demonstrated that GATA3 can induce morphological changes in 231 cells in 2D culture. In addition to changes in morphology in 2D, we observed changes in the structural organization of 231-GATA3 cells when cultured in 3D using Cultrex® basement membrane extract (BME). Elongated 231-Empty cells appeared invasive by protruding into the BME matrix with a tendency to form interconnected networks of cells, whereas the 231-GATA3 cells remained rounded, tightly organized clusters and failed to form extended protrusions through the BME (Figure 3B). These morphological changes promoted by GATA3 in 3D cell cultures are characteristic of a less invasive, more organized cell phenotype (Kenny *et al.*, 2007).

GATA3 has no effect on cell proliferation in 2D cultures but reduces proliferation in 3D cultures (task 1b)

GATA3 was shown to directly modulate expression of genes regulating the cell cycle (Pei *et al.*, 2009; Molenaar *et al.*, 2010) and GATA3 over-expression reduced proliferation in 293T cells (Usary *et al.*, 2004). Thus, we investigated whether GATA3 could alter proliferation of MB231 cells. Pulse-chase BrdU labeling revealed that GATA3 over-expression in MB231 did not affect proliferation in 2D cultures with 37% and 38% of cells in S-phase for 231-Empty and 231-GATA3 cells, respectively (Figure 4A). These findings are consistent with Yan *et al.* for 2D cultures (Yan *et al.*, 2010). However, in 3D Cultrex®, 231-GATA3 cells were significantly less proliferative compared to 231-Empty control cells ($p < 0.001$; Figure 4B). Thus, differences between 231-Empty and 231-GATA3 rates of cell proliferation may not necessarily be due to intrinsic cellular changes but may be the result of GATA3 altering the interaction of the cells with the extracellular matrix (ECM).

GATA3 reduces primary tumor outgrowth of xenografts (task 1c)

In xenograft studies, primary tumor outgrowth of 231-GATA3 cells was significantly delayed compared to 231-Empty cells when orthotopically transplanted (Figure 5A). Tumor growth of 231-GATA3 cells to a volume of 1 cm³ was delayed by 20 days compared to 231-Empty cells. Concomitant with these findings, we observed an improvement in cumulative survival of mice implanted with 231-GATA3 cells compared to those implanted with 231-Empty cells. Median survival was extended approximately 40% (from 53 to 73 days) for mice receiving

231-GATA3 cells compared to mice receiving the control cells (Figure 5B). Histologically, sections of 231-Empty tumors were characterized primarily by bundles and streams of spindylloid cells with scant to moderate eosinophilic fibrillar cytoplasm and large oval to elongate hyperchromatic nuclei with coarsely clumped marginated chromatin (Figure 5C). This spindylloid population was admixed with smaller numbers of round to ovoid neoplastic cells with scant eosinophilic amorphous cytoplasm and round to reniform eccentric hyperchromatic nuclei. Sections of tumors arising from 231-GATA3 cells displayed a less prominent spindylloid phenotype, with a predominance of cells exhibiting a round, or epithelioid appearance (Figure 5C). Often a spindylloid population was present centrally within GATA3 tumors, but it was not a predominant feature as compared to tumors arising from 231-Empty cells. Moreover, 231-GATA3 primary tumors stained positively for the luminal markers E-cadherin and cytokeratin 8 (Figure 5D) which was not observed for 231-Empty primary tumors, consistent with GATA3 inducing a more luminal differentiated phenotype.

GATA3 reduces metastatic potential of xenografts (task 1b and 1c)

In addition to orthotopic tumor growth, MB231 cells form metastatic lesions in the lung of tail-vein injected NOD/SCID mice. Thus, we examined the effect of GATA3 in altering the metastatic potential of MB231 cells *in vivo*. Our lab has previously demonstrated that metastatic burden in the lung from tail vein injected mice is proportional to fluorescent intensity by *ex-vivo* imaging (Barkan *et al.*, 2010). Although we did not observe a statistically significant difference in the number of 231-GATA3 cells compared to 231-Empty cells invading through Matrigel *in vitro* using the Boyden chamber assay (Figure 6A), there was a dramatic increase in the clearing of tail vein injected 231-GATA3 cells in the lungs compared to 231-Empty cells within the first 24 hrs following tail vein injection (Figure 6B). At 24 hours, there was an approximately 75% reduction in the number of 231-GATA3 cells in the lungs compared to the number of cells in the lungs 2 hours post-injection, whereas at the same time points there was an approximately 20% increase in the number of 231-Empty cells in the lungs (Figure 6B). This suggests that GATA3 greatly reduces the ability of MB231 cells to initially survive in the lung metastatic site. Furthermore, mice tail vein injected with 231-GATA3 cells had a statistically significant 9-fold reduction in total metastatic burden in the lung compared to mice injected with the 231-Empty cells 2-months after injection ($p < 0.05$; Figure 6C). The observed reduced metastatic burden in the lungs of mice receiving 231-GATA3 cells was the result of a reduced number and smaller size of lesions as observed by immunofluorescence (Figure 6D).

GATA3 reduces LOX expression in Hs578T cells and knock down of GATA3 in BT474 increases LOX expression (task 1a)

Microarray analyses revealed that the expression of numerous genes associated with cell adhesion, extracellular matrix remodeling, migration and metastasis were reduced in 231-GATA3 cells compared to 231-Empty cells which is consistent with the reduced metastatic propensity of the 231-GATA3 cells. One of those genes functionally involved in all of these processes is LOX, which was the gene most down-regulated by GATA3 by microarray analysis. Since LOX was shown previously to be involved in breast cancer metastasis to the lung and in

tissue remodeling (Erler *et al.*, 2006; Erler *et al.*, 2009), we investigated whether the dramatic reduction in metastatic propensity of 231-GATA3 cells was the result of GATA3 dependent inhibition of LOX expression. Reduced LOX expression in 231-GATA3 cells was confirmed by Q-RT-PCR. LOX expression was reduced by 70% in 231-GATA3 cells compared to 231-Empty cells ($p<0.01$; Figure 7A). When GATA3 was expressed in another BTNBC cell line, Hs578T, LOX expression was reduced by 30% ($p<0.05$; Figure 7A). Furthermore, 231-GATA3 cells had significantly reduced LOX catalytic activity compared to 231-Empty cells, consistent with the reduction in LOX expression ($p<0.01$; Figure 7B).

To further confirm that GATA3 regulates LOX expression in breast cancer cells, we knocked-down GATA3 expression in the luminal, GATA3-positive breast cancer cell line BT474 and measured LOX expression. We initially used a lentivirus construct expressing shRNA for GATA3. We used 5 different RNAi sequences and sorted cells after transduction. The siRNA sequence that resulted in best knock down of GATA3 also reduced slightly the proliferation of cells. However, as we passaged the cells, we observed loss of knock down and GATA3 was reexpressed in our knock down cells. There was a strong selection against the knock down of GATA3. Thus, we decided to do transient transfections with siRNA for GATA3. Knock-down of GATA3 was successfully achieved with two different siRNA sequences resulting in a 76% and 75% reduction in GATA3 expression for siGATA#2 and siGATA#3F, respectively. LOX expression was increased 4-fold and 4.5-fold with siGATA#2 and siGATA#3F, respectively (Figure 7C). These findings suggest that GATA3 can regulate LOX expression in both basal and luminal breast cancer subtypes.

KEY RESEARCH ACCOMPLISHMENTS

Overexpression of the transcription factor, GATA3 in MDA-MB-231 cells (231-GATA3) results in a more luminal phenotype and a concomitant reduction in the expression of metastasis-associated genes compared to control 231-Empty cells.

231-GATA3 cells showed altered morphology and cytoskeletal organization in 2D culture and in 3D cultrex

231-GATA3 and 231-Empty cells have similar proliferation rates in 2D but 231-GATA3 shows reduced proliferation in 3D cultrex compared to 231-Empty

Orthotopic implantations of 231-GATA3 cells showed reduced primary tumor growth in xenografts compared to 231-Empty cells

231-GATA3 cells showed similar invasive potential by Boyden chamber compared to 231-Empty but we observed reduced metastatic potential in xenografts compared to 231-Empty cells

231-GATA3 reduces LOX expression in MDA-MB-231 and in another triple negative breast cancer cell line, Hs578T.

Transient knock down of GATA3 in the luminal GATA3 positive BT474 cell lines, increases GATA3 expression.

REPORTABLE OUTCOMES

Oral Presentations

1. **Isabel Chu**, Aleksandra M. Michalowski, Mark Hoenerhoff, Kornelia M. Szauter, Dror Luger, Misako Sato, Akira Oshima, Katalin Csiszar and Jeffrey E. Green. GATA3 Induces Luminal Reprogramming of Human Basal Triple Negative Breast Cancer Cells and Inhibits Metastases through Suppression of Lysyl Oxidase. 2011. Center for Cancer Research Fellows Young Investigators Colloquium, Williamsburg, Virginia.
2. **Isabel Chu**, Aleksandra Michalowski and Jeffrey Green. Trans-differentiation of breast cancer by GATA3 reduces primary growth and metastasis. 2010. Center for Applied Medical Research, University of Navarra, Pamplona, Spain.

Abstracts

1. **Isabel M. Chu**, Aleksandra Michalowski and Jeffrey Green. Trans-differentiation of breast cancer by GATA3 reduces primary growth and metastasis. Frontiers in Tumor progression. Oct 24-28, 2010. Madrid, Spain

Microarray database

We deposited in GEO the microarray database for 231-Empty and 231-GATA3 <http://www.ncbi.nlm.nih.gov/projects/geo/query/acc.cgi?acc=GSE24249>].

CONCLUSION

The work accomplished on the first year of the proposed grant identified a key mechanism for the trans-differentiation of BTNBC to a more luminal phenotype through the expression of GATA3 resulting in inhibition of metastatic propensity of this aggressive form of breast cancer. We have demonstrated that the expression of GATA3 induces global changes to the transcriptome resulting in major phenotypic changes involving cellular differentiation, alterations in interactions with the extracellular microenvironment, paracrine signaling and metastatic propensity. GATA3 reprogrammed BTNBC cells to express more luminal differentiation markers and display luminal cellular biological properties. Importantly, GATA3 expression greatly diminished the aggressive nature of the BTNBC cells, significantly reduced their metastatic propensity and extended the survival of mice in xenograft studies. My work identified that GATA3 can reduce LOX expression, a gene involved in metastasis.

Taken together, our data suggest that GATA3 alone is sufficient to alter molecular events that can regulate both luminal and basal tumor differentiation and metastasis and most importantly suggest that the same factors that associate with differentiation status and subtype clustering are factors that control metastatic propensity with LOX as a prime example. This provides an important link between cellular differentiation (i.e., basal vs. luminal) and metastasis through the transcription factor GATA3. Thus, GATA3 behaves as a master regulator controlling both differentiation and invasiveness.

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SUPPORTING DATA

FIGURES

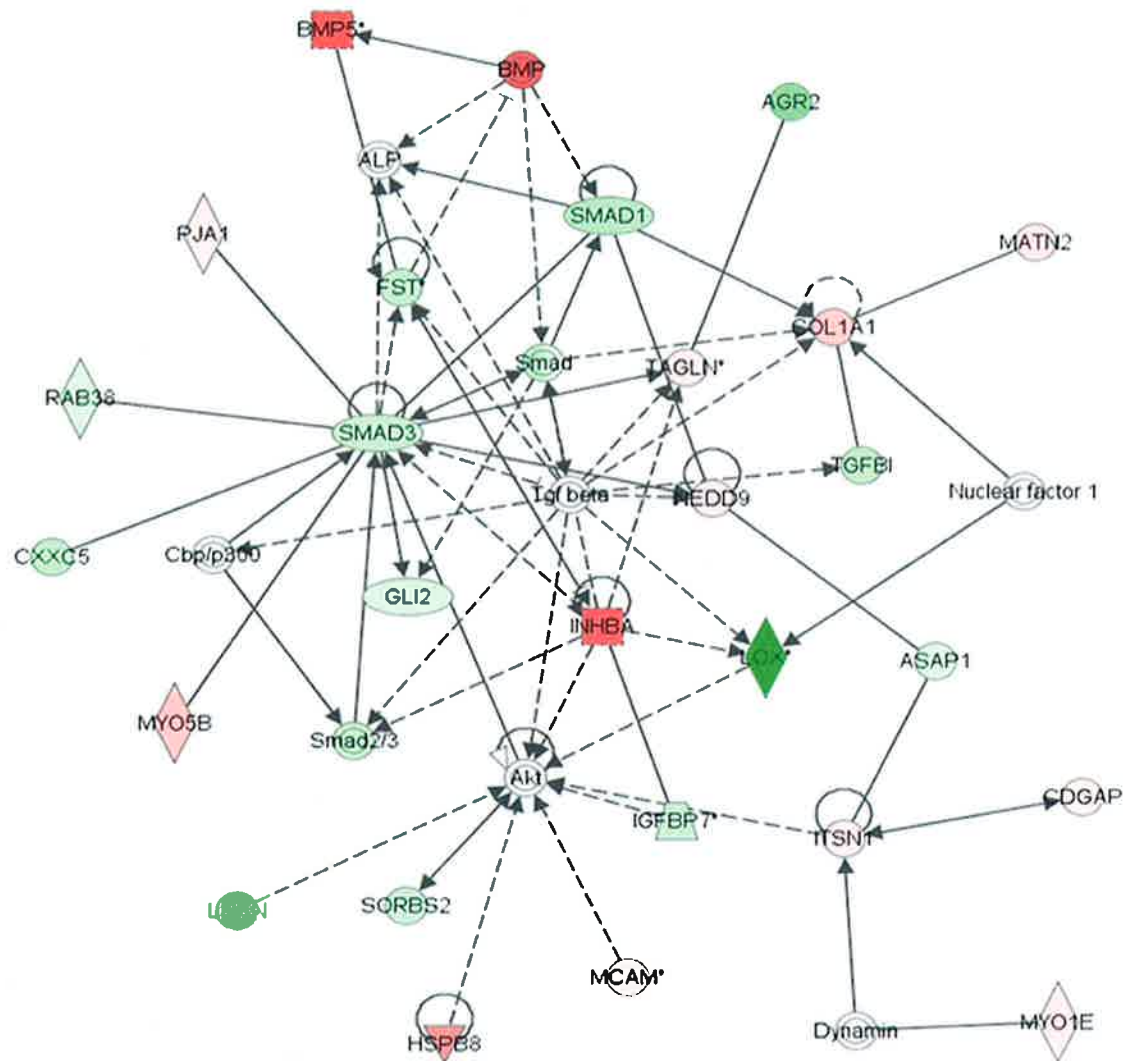


Figure 1. Ingenuity pathway analysis of 231-Empty and 231-GATA3. 231-Empty and 231-GATA3 cells were analysed by microarray and genes that were differentially expressed were input in Ingenuity. Genes in Green are downregulated in 231-GATA3 cells and genes in red are upregulated. 231-GATA3 reduces genes in LOX and TGF-beta pathway.

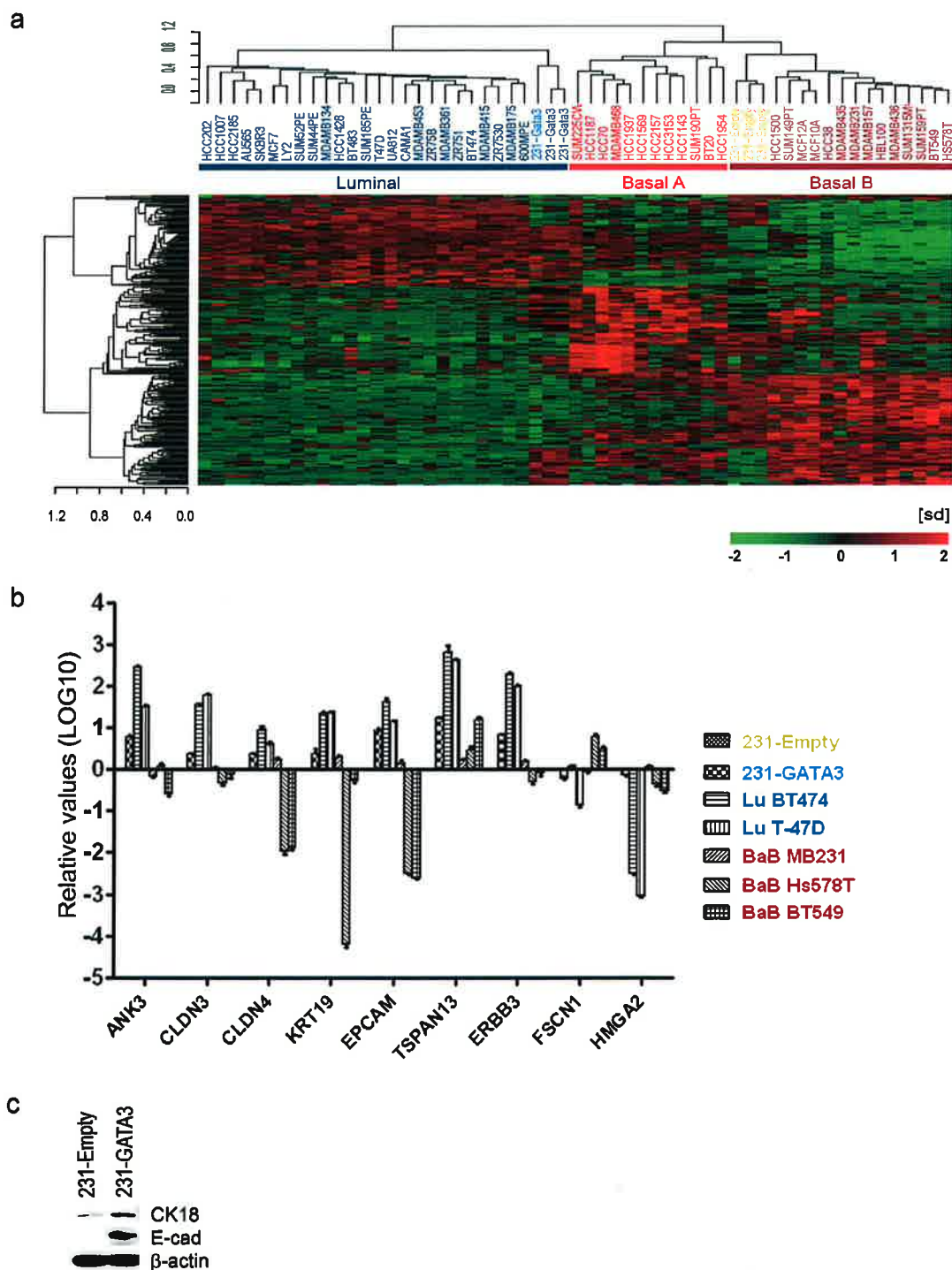


Figure 2. Microarray analysis of 231-Empty and 231-GATA3 cells. (a) Hierarchical cluster analysis of 51 breast cancer cell lines (Neve et al., 2006) and the 231-Empty and 231-GATA3 cells. Two hundred forty nine unique genes from the subtype predictor signature (Neve et al., 2006) are included in the analysis (multiple microarray probes per gene reduced to the probe with the highest median intensity across samples). The heatmap gene expression values are relative differences within the respective datasets (gene-wise z-score transformation in the 51 cell line collection and the 231-Empty and 231-GATA3 cells). The two-way hierarchical clustering of the combined data sets employs distance metric of one minus Pearson's correlation coefficient and average linkage algorithm. (b) Q-RT-PCR validation of changes in gene expression in the 231-Empty vs. 231-GATA3 cells. (c) Western blot showing CK18 and E-cadherin in 231-Empty vs. 231-GATA3. β -actin was used as a loading control. There is re-expression of E-cadherin and increased CK18 in 231-GATA3 vs. 231-Empty cells.

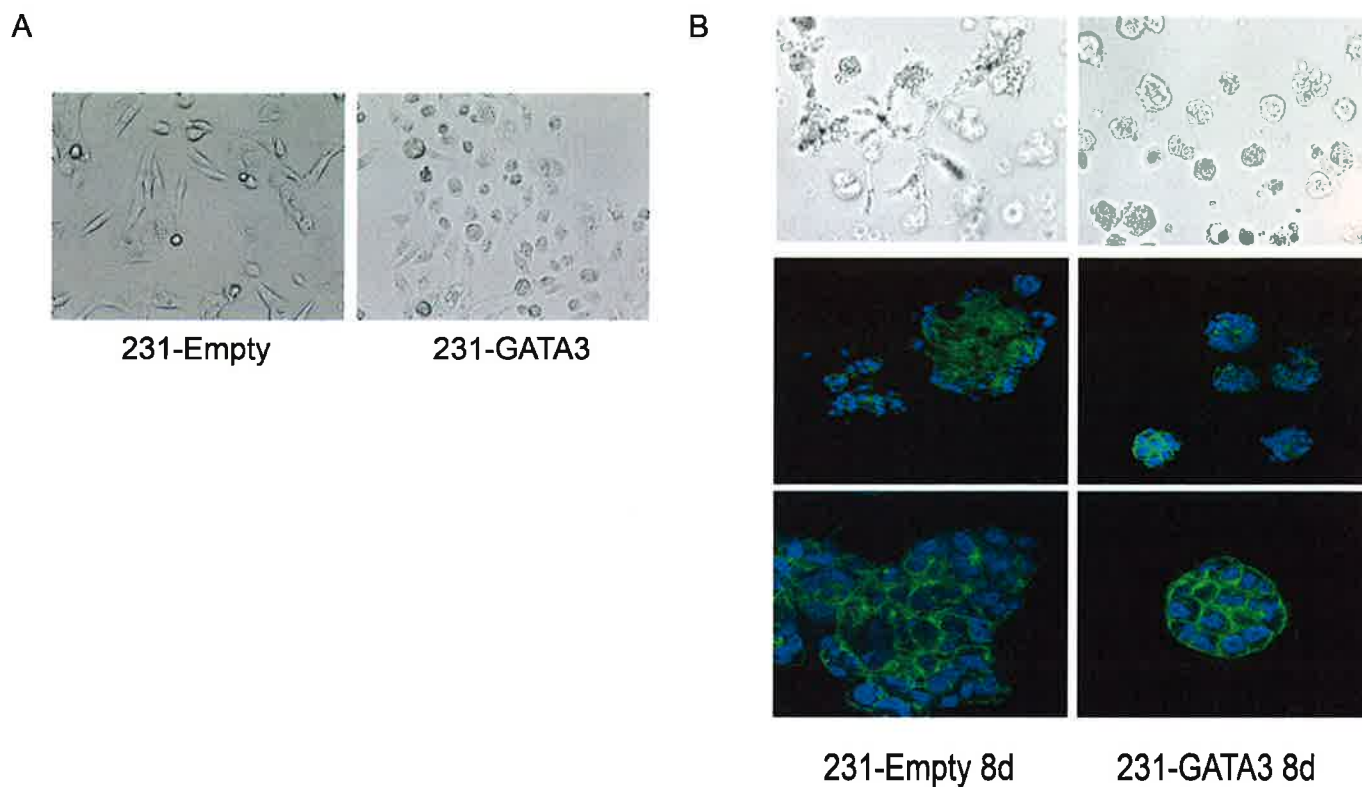
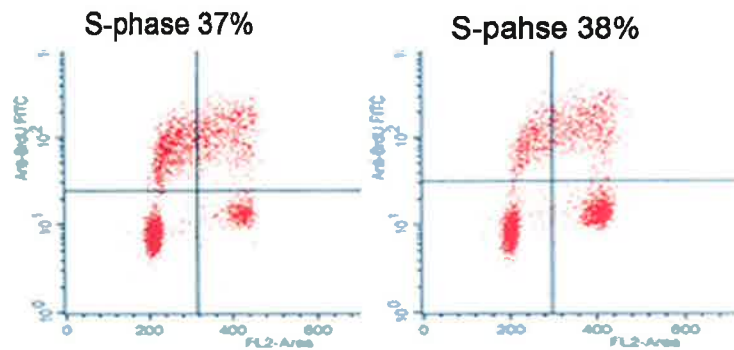


Figure 3 GATA3 overexpression in MB231 promotes morphological changes in 2D culture and 3D cultrex. (a) Bright field images of 231-Empty and 231-GATA3 on 2D culture. 231-GATA3 cells became cuboidal while 231-Empty cells retained their spindle elongated morphology. (b) Top panels, bright field images; lower panel, confocal microscopy of cells on 3D Cultrex® fixed and stained with DAPI (blue) for nuclear localization and phalloidin (green) for f-actin. 231-GATA3 formed more organized structures, however, 231-Empty cells formed elongated protrusions.

a



b

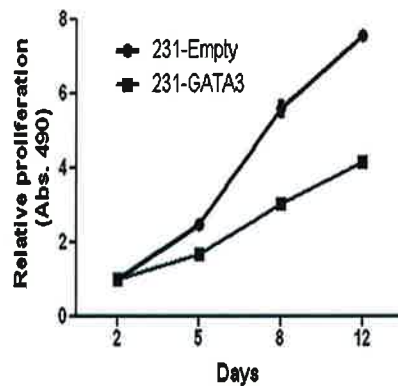


Figure 4. 231-GATA3 cells show reduced proliferation in 3D but no changes in 2D cultures.. (a) Flow cytometric analysis showing cell cycle profile of 231-Empty vs. 231-GATA3 cells. There is no difference in %S-phase in both cells in 2D culture. b) 231-Empty and 231-GATA3 cells were seeded on 3D Cultrex®. 231-GATA3 cells show reduced proliferation as measured by MTS (mean +/- SEM) compared to 231-Empty

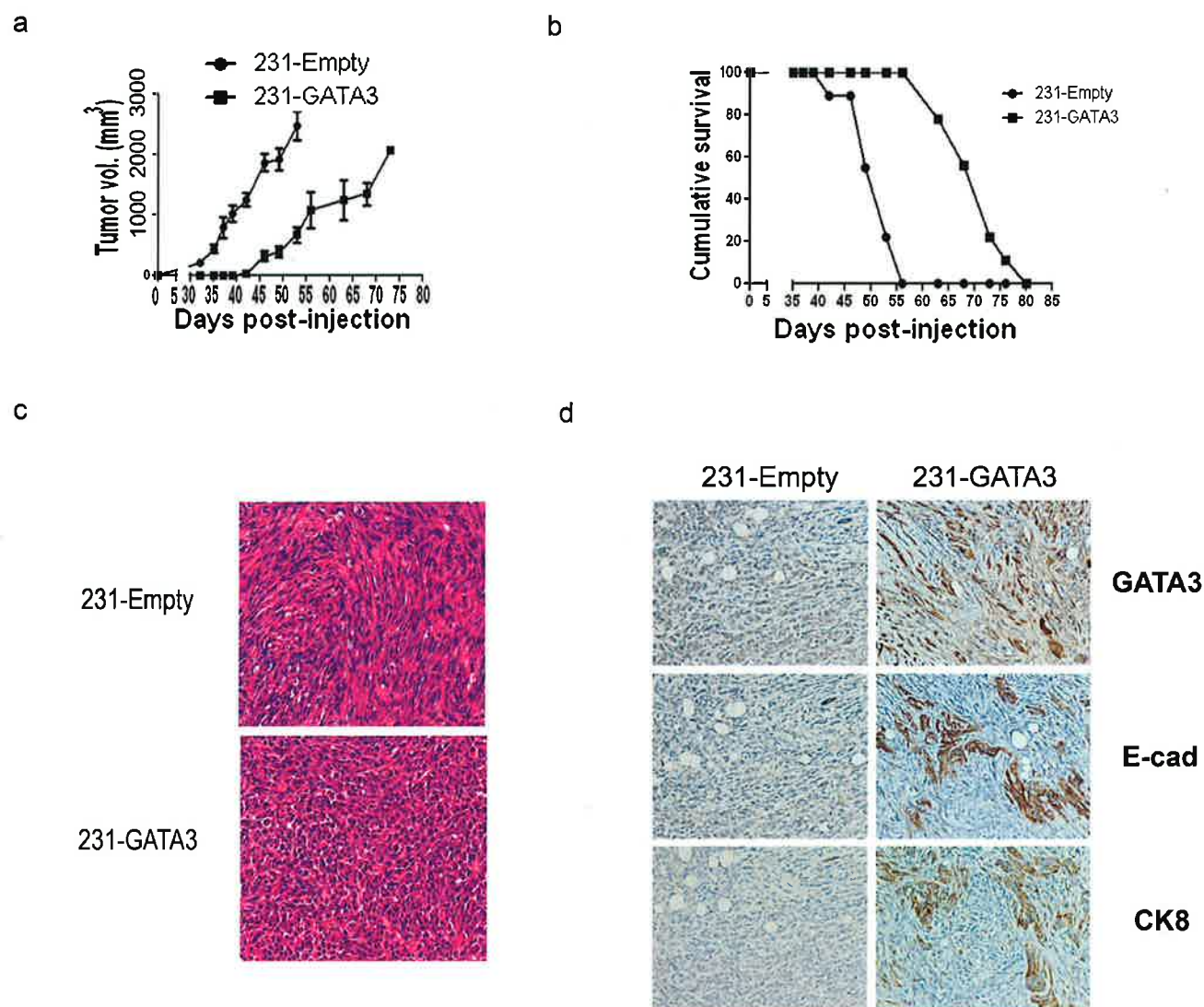


Figure 5. Orthotopic implantation of 231-Empty and 231-GATA3 cells. (a) 231-GATA3 exhibit reduced primary tumor growth compared to 231-Empty cells (tumor size measured every 2-4 days (vol.=l x w2 x 0.4). (b) increased survival of mice receiving 231-GATA3 vs. 231-Empty cells. Mice were euthanized when the tumor reached 2 cm in diameter. (c) Primary 231-GATA3 mammary tumor xenografts display a more prominent epithelioid phenotype compared to a pre-dominant spindly appearance of 231-Empty mammary xenografts. H&E staining. (d) Immunohistochemical staining of primary tumors confirmed positive staining for GATA3 in only 231-GATA3 tumors. There was an association of E-cadherin and CK8 staining with GATA3 expression in 231-GATA3 tumors. 231-Empty tumors were negative for GATA3.

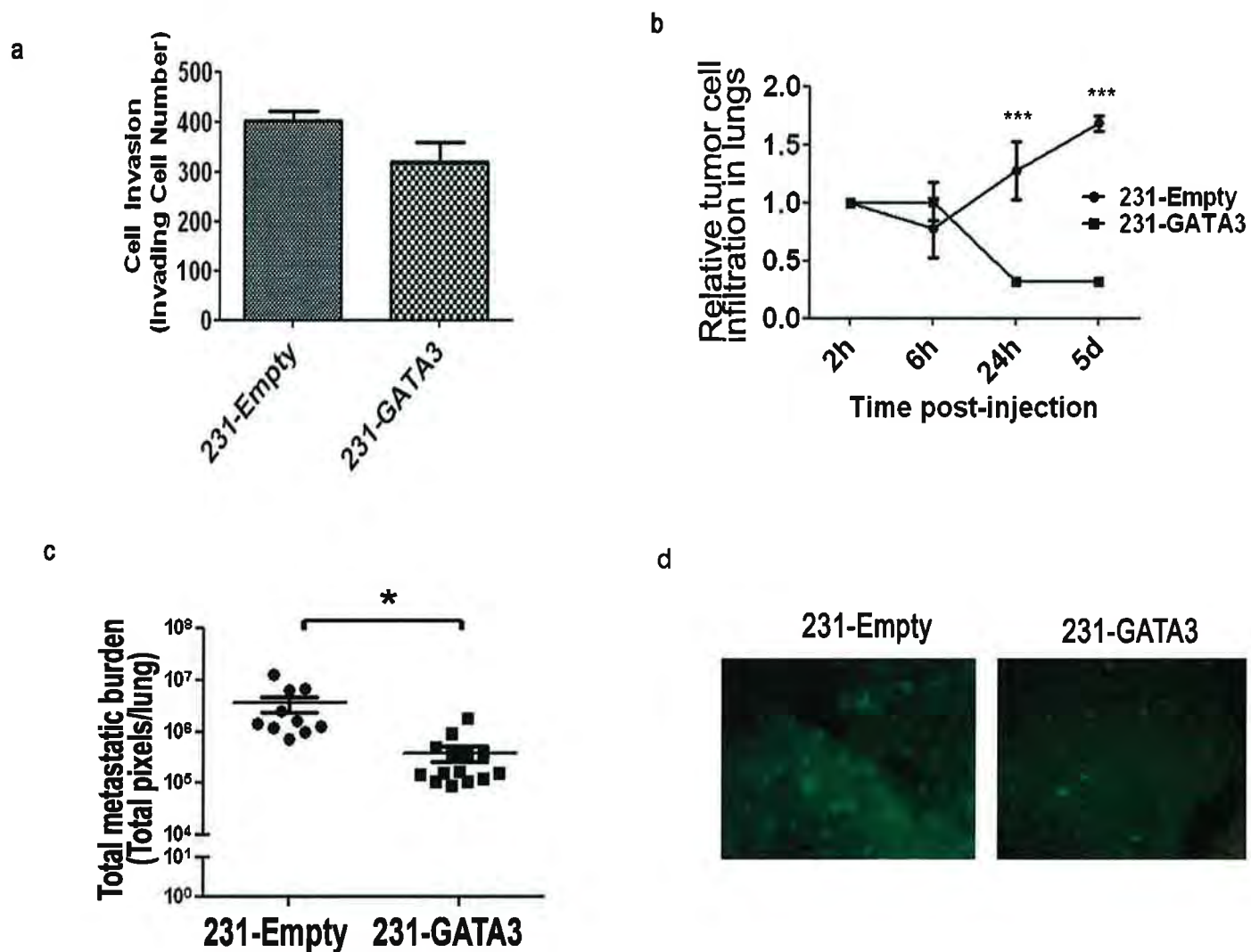
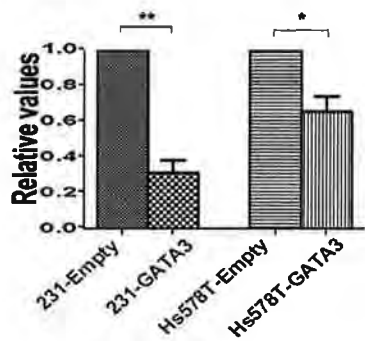
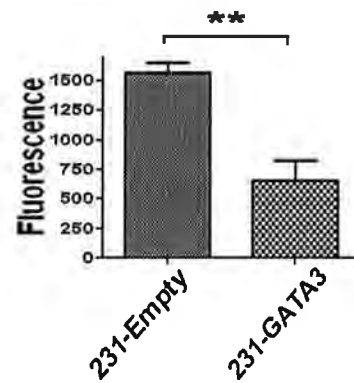


Figure 6. Reduced lung metastases in mice receiving 231-GATA3 cells by tail vein injection compared to 231-Empty cells. (a) Boyden Chamber assay of 231-Empty and 231-GATA3 cells showed a non-statistically significant trend for reduced number of cells invading through a Matrigel coated chamber. (b) Relative tumor cell infiltration in lungs as measured by immunofluorescence of lungs from tail vein injected mice with 231-Empty or 231-GATA3 cells expressing GFP. Lungs were collected at the indicated times. (c) Lung lesions of mice injected by tail vein with 231-Empty and 231-GATA3. Lungs were imaged by fluorescent microscopy with total metastatic burden calculated per lung. (d) Immunofluorescence picture of the lung of 231-Empty and 231-GATA3 tail vein injected mice

a



b



c

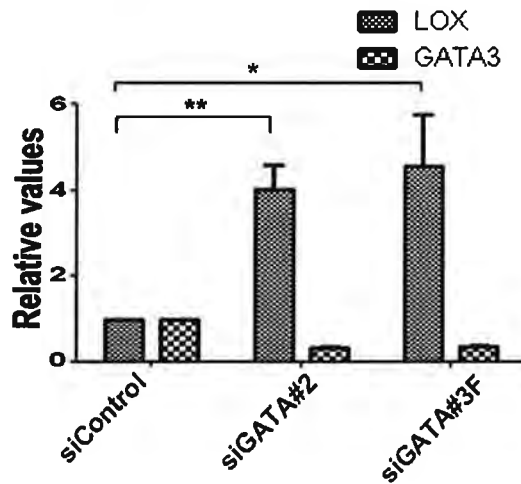


Figure 7 GATA3 regulates LOX expression in breast cancer cells. (a) Relative LOX expression by Q-RT-PCR. Samples were normalized to cyclophilin. Over-expression of GATA3 in MB231 and Hs578T cells reduces LOX mRNA expression. (b) Relative LOX activity in the media of 231-Empty and 231-GATA3 cells measured as the increase in fluorescence over BAPN containing controls. Relative activity measured at 2400 seconds (40 min). (c) Relative LOX and GATA3 mRNA expression measured by Q-RT-PCR. BT474 cells were transfected with siRNA for GATA3 for 72 hrs prior to RNA isolation.